PULLULANASE FROM AEROBACTER AEROGENES; PRODUCTION IN A CELL-BOUND STATE. PURIFICATION AND PROPERTIES OF THE ENZYME

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Some years ago we isolated a strain of Aerobacter aerogenes capable of growing with pullulan, an extracellular α-glucan of the yeastlike fungus Pullularia pullulans, as the sole source of carbon (Bender and Wallenfels 1961). The enzyme used to hydrolyze the polymer was shown to be a rather specific 1,6-α-D-glucosidase splitting pullulan almost quantitatively to maltotriose (Bender and Wallenfels 1961, Wallenfels et al. 1965). The enzyme, which was named "pullulanase", is formed by A.aerogenes only under induction, and is released from the cells in the later logarithmic growth phase. Besides pullulan, maltose and maltotriose have been found to be effective inducers. Attempts to purify the enzyme from preparations obtained by acetone-precipitation from the culture filtrate had only partly been successful (Freudenberger 1962).

We have found that the release of the pullulanase into the culture filtrate can be prevented by specific culture methods. It proved to be much easier to purify the enzyme from a preparation obtained by specific extraction of the cells than from

the culture filtrate. As far as we can tell at present, no differences exist between the cell-free and the cell-bound pullulanase.

EXPERIMENTAL AND RESULTS

A. Enzyme Assay. The assay method originally described (Bender and Wallenfels 1961, Wallenfels et al. 1965) has been somewhat modified. The unit of activity is defined as that amount of enzyme, which at 30°C and pH 5.0, liberates one µmol of maltotriose in one minute. The increase in reducing power is measured with Nelson reagent (Nelson 1944). Pure maltotriose is used for calibration.

B. Specific Culture Methods. If Aerobacter is grown in the mineral Czapek-medium (K_2HPO_4 0.1%, $NaNO_3$ 0.3%, $MgSO_4.7\ H_2O$ 0.05%, KCl 0.05%, $FeSO_4.7\ H_2O$ 0.001%; pH 7.2) with 0.8% maltose as the source of carbon, the synthesis of pullulanase begins after a lag of about three hours, together with growth, and parallels the increase of cell mass (fig.1). During the first hours of the exponential growth, the enzyme is totally cell-bound. Cell-free activity appears in the later logarithmic growth phase, and increases up to 75% - 80% of the total. The amount of cell-bound enzyme correspondingly decreases with the increase of extracellular one.

Quite another behaviour of the enzyme is found, if maltose and glucose together (each 0.4% in concentration) serve as the sources of carbon (fig.2). Apparently, a special form of diauxy (Monod 1942) occurs: Aerobacter first uses the glucose, as can be shown by chromatography, but, in addition, half of the maltose also disappears from the medium in this period of growth. The synthesis of pullulanase is, however, completely

inhibited. Without a marked lag in growth, but with a marked

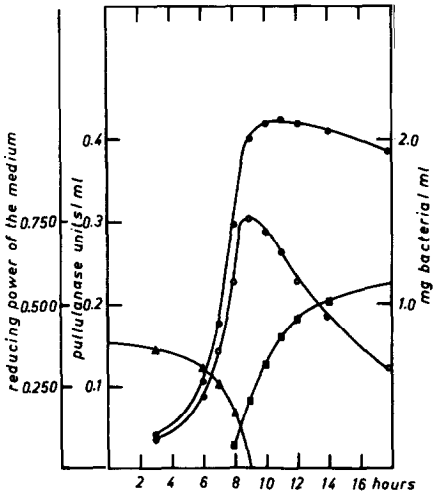

Fig.1

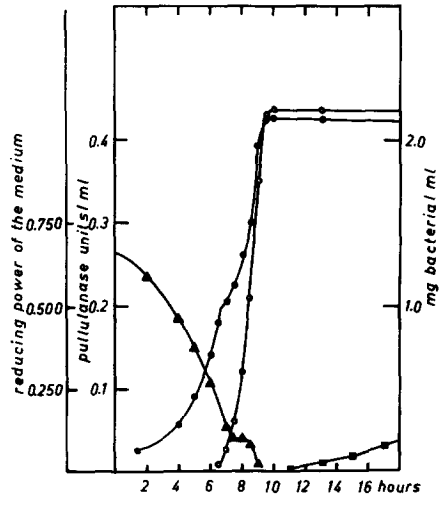

Fig.2

Pullulanase production by Aerobacter aerogenes

Fig.1. Growing on 0.8% maltose as the sole source of carbon
Fig.2. Growing on 0.4% maltose and 0.4% glucose.
Full circles, bacterial dry weight; open circles, cell-bound pullulanase; squares, extracellular pullulanase; triangles, reducing power of the growth medium (arbitrary units). To measure the cell-bound activity, the cells were harvested by centrifugation, washed with distilled water, resuspended in M/100 citrate phosphate buffer, pH 5.0, and disrupted in an ultrasonic oscillator.

period in which the sugar content of the medium remains constant, the enzyme production is started with the sole utilization of maltose. The activity increases, corresponding to growth, within three hours to the maximal level. In comparison with the cultivation on maltose alone, the enzyme is not released from the cells. Even after prolonged incubation only 20% of the total activity become cell-free. As the intact cells are as active as cell-homogenates, the pullulanase behaves now like a surface enzyme (Pollock 1962).

In a similar way pullulanase release is inhibited by growing Aerobacter in continuous culture. For continuous cultivation the Czapek-medium is used. Growth limiting factor is

the carbon source, e.g. 0.25% maltose, or 0.25% pullulan, or 0.2% glycerol, together with 0.08% maltose or 0.08% pullulan for induction. To start the continuous run, Aerobacter is allowed to grow on maltose and glucose (each 0.4% in concentration), in order to obtain the enzyme in a cell-bound state from the beginning of the cultivation. The continuous addition of medium is started at the end of the exponential growth. A dilution rate of 0.2 hours^{-1} has proved to be optimal for pullulanase production.

C. Specific Extraction. Pullulanase, produced under the conditions described, is very tightly bound. Even prolonged shaking of the resuspended cells in water, different buffers, or hypertonic solutions, removed only 1/3 of the cell-bound activity. By intensive mechanical disintegration, all pullulanase is set free. The specific activity of these extracts, however, is very low.

As the enzyme in cell-bound state seems to be localized near the cell surface, detergents were chosen to bring about the liberation of the enzyme. These substances have not only been used for liberation of ribosome-bound enzymes (Zipser 1963), but it was also shown that they effect cytoplasmic membranes (Nermut 1964), and isolated cell walls (Martin and Frank 1962, Weidel et al. 1963).

Indeed, nearly all the pullulanase can be extracted, more or less specifically, by shaking the cells in aqueous solutions of various detergents. The best results were obtained with sodium-laurylsulphate (SDS), or the covalent triton-X 100. 90% - 95% of the pullulanase can be extracted, if 10g of fresh wet cells are shaken for 15 hours at 30°C in either 100 ml of

a 0.1% (w/v) solution of SDS or a 0.4% (w/v) solution of triton-X 100.

D. Purification. The experiments described below were carried out with SDS-extracts, The triton-X 100 extracts, however, behave similarly. Only an approximately 4-fold purification of the pullulanase, obtained with these extracts, has proved to be necessary for obtaining pure protein. The purification is performed in two steps.(table 1).

1. DEAE-adsorption: 500 ml of the enzyme solution, obtained by SDS-extraction of 50 g wet cells, containing 3,500 pullulanase units and 170 mg total protein, are mixed with a suspension of 3.0g DEAE-cellulose in 50 ml of a 0.01 M Tris-HCl buffer, pH 7.7, and stirred for 30 minutes at 0°C. The cellulose is removed, and washed twice with a 0.1 M $(NH_4)_2SO_4$-solution. The desorption of the enzyme is performed by stirring the cellulose in 20 ml of a 0.4 M $(NH_4)_2SO_4$-solution for 30 minutes. The procedure is repeated once again with another 20 ml of the salt solution. 2,800 units (80% of the activity) in 81.6 mg protein are recovered.

2. $(NH_4)_2SO_4$-fractionation: Saturated $(NH_4)_2SO_4$-solution is added dropwise at 0°C to 40 ml of the enzyme solution obtained by step 1. Precipitates formed at saturations of 0.05 - 0.33 (I), 0.33 - 0.50 (II), and 0.50 - 0.64 (III) are removed by centrifugation, and dissolved in 4 ml of 0.002 M phosphate buffer, pH 7.2. Fraction I contains 18 units and 1 mg protein (material other than protein is also precipitated), fraction II 2,100 units and 27.2 mg protein, and fraction III 420 units and 21 mg protein. In connection with the previous step, 90% of the enzyme and 60% of the protein are recovered. 82% recovery

Table 1. Purification procedure of cell-bound pullulanase from continuously cultivated cells (0.4 - 0.6 units/ ml culture suspension).

procedure	volume(ml)	units/ml	units(total)	mg protein/ml	spec.activity	recovery[1)]
SDS-extract	500	7	3,500	0.340	20	-
DEAE-adsorption	40	70	2,800	2.04	35	80%
$(NH_4)_2SO_4$-fractionation						
I 0.05-0.33 saturation	4	4.5	18	0.250	18	
II 0.33-0.50 "	4	525	2,100	6.8	76	90%
III 0.50-0.64 "	4	105	420	5.2	20	

1) in connection with the previous step

is obtained with fraction II, with a specific activity of 76 units /mg protein.

E. Properties Of Purified Pullulanase. Preliminary analytical studies were carried out with fraction II, after dialysis against 0.002 M phosphate buffer, pH 7.2, for 48 hours at 0°C. Electrophoresis on polyacrylamide-gel shows only one band, if stained with amidoblack 10B, indicating the presence of a single protein (fig.3). In the sedimentation diagram, a second

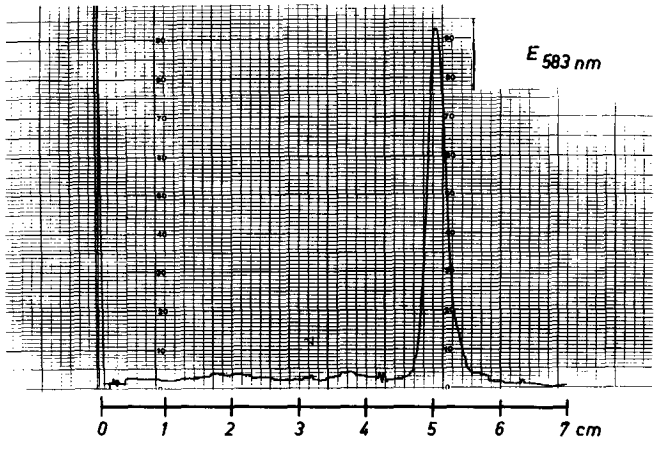

Fig.3. Electrophoretogram of pullulanase. The electrophoresis was carried out as described elsewhere (Raymond and Wang 1960, Raymond and Nakamichi 1962), on 5% polyacrylamide-gel in 0.05 M Tris-HCl buffer, pH 7.6, with a horizontal apparatus, at 40 mA for 4 hours. Cooling was performed withrunning water. The electrophoretogram was stained with amidoblack 10B, and the colour intensity was measured with a recording photometer at 583 mμ. The pullulanase moves to the anode.

small peak can be seen, corresponding to a slower-moving component (fig.4). After separation, it was shown to be biuret-negative and strongly anthrone positive, without any pullulanase activity. The sedimentation and diffusion data, obtained with the main peak, containing all activity, point to a molecular weight of approximately 145,000 for the pullulanase protein.

Fig.4. Sedimentation of pullulanase in M/15 phosphate buffer, pH 7.2 (6.8 mg protein/ml) at 20°C. The course of sedimentation was followed in a valvetype cell (angle 60°) at 59,780 rpm. The photos were taken at intervals of 8 minutes.

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